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Polyphenols Selectively Reverse Early-Life Stress-Induced Behavioural, Neurochemical and Microbiota Changes in the Rat

Francisco Donoso[a, b], Sian Egerton[a, d, e], Thomaz F. S. Bastiaanssen[a, c], Patrick Fitzgerald[a, c], Snehal Gite[d], Fiona Fouhy[d], R. Paul Ross[a], Catherine Stanton[a, d], Timothy G. Dinan[a, b] and John F. Cryan[a, c]

[a]APC Microbiome Ireland, University College Cork, Cork, Ireland
[b]Department of Psychiatry & Neurobehavioural Science, University College Cork, Cork, Ireland
[c]Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[e]School of Biological, Earth and Environmental Science, University College, Cork, Ireland.

Correspondence: Prof. John F. Cryan, Dept. Anatomy & Neuroscience/APC Microbiome Ireland, University College Cork, Ireland (j.cryan@ucc.ie)

Highlights

- Dietary polyphenols prevented depressive- and anxiety-like behaviours in rats exposed to early life stress.
- A polyphenolic diet ameliorated a dysregulation of the HPA axis and BDNF levels in rats subjected to early life stress.
- Dietary polyphenols reversed early life stress-induced changes in gut microbiota composition.
- Polyphenol-driven improvements in behaviour and physiology may be mediated by modulation of the microbiota-gut-brain axis.

Abstract

There is a growing emphasis on the role of the microbiota-gut-brain axis as modulator of host behaviour and as therapeutic target for neuropsychiatric disorders. In addition, accumulating evidence suggests that early-life stress can exert long-lasting changes on the brain and microbiota, and this early adversity is associated with increased risk for developing depression in later life. The maternal separation (MS) model in rats is a robust paradigm to study the effects of early-life stress on the microbiota-gut-brain axis. Recently, we have shown that polyphenols, naturally occurring compounds associated with several health benefits, have anti-stress effects in *in vitro* models. In this study, we assess the therapeutic potential of a variety of both flavonoid and non-flavonoid polyphenols in reversing the impact of MS on behaviour and the microbiota-gut-brain axis.

Rats underwent a dietary intervention with the naturally-derived polyphenols xanthohumol and quercetin, as well as with a phlorotannin extract for 8 weeks. Treatment with polyphenols prevented the depressive and anxiety-like behaviours induced by MS, where xanthohumol effects were correlated with rescue of BDNF plasma levels. In addition, MS resulted in altered brain levels of 5-hydroxyindoleacetic acid (5-HIAA) and dopamine, accompanied by abnormal elevation of plasma corticosterone. Although polyphenols did not reverse neurotransmitter imbalance, xanthohumol normalised corticosterone levels in MS rats. Finally, we explored the impact of MS and polyphenolic diets on the gut microbiota. We observed profound changes in microbial composition and diversity produced by MS condition and by xanthohumol treatment. Moreover, functional prediction analysis revealed that MS results in altered enrichment of pathways associated with microbiota-brain interactions that are significantly reversed by xanthohumol treatment. These results suggest that naturally-derived polyphenols exert antidepressant-like effects in MS rats, which mechanisms could be potentially mediated by HPA regulation, BDNF levels rescue and modulation of the microbiota-gut-brain axis.

Keywords: *Polyphenols; Microbiota-Gut-Brain Axis; Early-life Stress*

1. Introduction

Stress-related psychiatric disorders including depression and anxiety are currently a major public health concern. Indeed, the World Health Organisation (WHO) has predicted that depression will be the second largest cause of disability by 2020 (Johnston et al., 2019). On the other hand, major depressive disorder is thought to result from the complex interplay of multiple inherited genetic factors and subsequent exposure to a wide range of environmental variables throughout life (aan het Rot et al., 2009); therefore, the search for adequate treatments is a great challenge as no established mechanisms have yet been determined (Berton and Nestler, 2006). Based on these observations and considering that depression has an inconsistent response to treatment, the development of new antidepressant strategies is increasingly being considered as a critical focus of research.

It is well known that stressful events in early life can exert long-lasting changes in brain structure and function later on (Cryan and Dinan, 2013) and accumulating evidence indicates that this early life adversity is associated with an increased risk for developing depression (Chapman et al., 2004; Heim and Binder, 2012). For instance, inadequate maternal care has been linked to developmental, emotional and social deficits in humans (Field, 1998). In rodents, the maternal separation (MS) model is a well-described paradigm used to investigate the neurobiological and behavioural consequences of early life stress (Nishi et al., 2014; O'Mahony et al., 2011; Rincel and Darnaudery, 2019). For this reason, the MS model has been used to study various psychiatric conditions, especially depression (Vetulani, 2013)(Meaney et al., 1996) (Wieck et al., 2013).

The microbiota-gut-brain axis describes the complex bidirectional communication system that exists between the central nervous system (CNS) and enteric microbiota; involving endocrine, immune and neural pathways (Cryan et al., 2019; Foster et al., 2017; Rhee et al., 2009). Accumulating research has focused on the impact of the microbiota on CNS function and stress perception, and its consequences for behaviour (Cryan and Dinan, 2012). Indeed, top down activation of the CNS can influence gut neuromotor and secretory function, immunity and microbiota composition during stress (De Palma et al., 2014; Foster et al., 2017). In this regard, early-life stress models such as MS have long-term impact on the gut microbiota, which correlate with increased HPA axis activity and behaviour (Bailey and Coe, 1999; O'Mahony et al., 2009). Moreover, the MS model is sensitive to reversal treatments that target the gut microbiota (Cowan et al., 2019; Fukui et al., 2018; Gareau et al., 2007; McVey Neufeld et al., 2019; O'Mahony et al., 2019).

The emerging and compelling evidence for nutrition as a crucial factor in the high prevalence and incidence of mental disorders suggests that changes in diet are a viable strategy for improving mental health and treatment of psychiatric disorders including anxiety and depression (Adan et al., 2019; Dinan et al., 2019; Jacka et al., 2014; Lai et al., 2014; Larrieu and Laye, 2018; Spencer et al., 2017). For instance, dietary polyphenols are a group of naturally occurring phytochemicals which are present in high amounts in fruits and vegetables and are characterised by the presence of multiple hydroxyl groups on aromatic rings (Vauzour, 2012). Several studies have focused on the potential of polyphenolic compounds in protecting cognitive function and reducing risk for developing neurodegenerative disorders (Spencer, 2008). In particular, some pre-clinical studies have confirmed the antidepressant capacity of polyphenols in different animal models (Anjaneyulu et al., 2003; Kulkarni et al., 2008; Yi et al., 2008). Moreover, dietary polyphenols are capable of modulating the composition of the gut microbial community by inhibiting or stimulating the growth of certain bacteria (Lee et al.,

2006). Hence, there is increasing interest in using polyphenols to target the microbiota-gut-brain axis to treat mental disorders (Filosa et al., 2018; Matarazzo et al., 2018).

Polyphenolic compounds are characterised as having different functional activity depending on their chemical structure (Manach et al., 2004; Vauzour et al., 2010). For instance, phlorotannins are a type of polyphenolic tannins found in marine brown algae, which have been shown to possess anti-oxidant activity, as well as beneficial effects for different diseases such as cancer, cardiovascular problems and diabetes (Kim and Himaya, 2011). Other polyphenols can only be isolated from specific sources. Xanthohumol, for example, is described as a prenylated chalcone, a principal component of the female hop plant, *Humulus lupulus* (Stevens and Page, 2004). Some health benefits associated with xanthohumol intake include anti-inflammatory and neuroprotective effects (Liu et al., 2015). In contrast, some members of the flavonoid family like quercetin are widely distributed in nature (Manach et al., 2004). Quercetin is one of the most studied polyphenols and has been demonstrated to confer protection against certain types of cancer, cardiovascular and neurodegenerative disorders (Boots et al., 2008).

Recently, we showed that across a wide number of polyphenols, xanthohumol and quercetin were able to reverse the impact of corticosterone exposure in primary cortical neurons (Donoso et al., 2019). Moreover, although the antidepressant effects of several polyphenols have been studied in different preclinical studies (Bhutada et al., 2010; Liu et al., 2014; Xu et al., 2005), their therapeutic effects have not yet been examined in models of early life stress, nor the mechanisms underlying the polyphenol-mediated alleviation of mood. Therefore, the purpose of this study was to explore the therapeutic effects of different naturally derived polyphenols, including phlorotannins, xanthohumol and quercetin in the MS model in rats. In addition, the

consequences of MS and polyphenol diet intervention on different aspects of the microbiota-gut-brain axis were explored.

In this regard, we evaluated important components involved in the regulation of this axis, including BDNF, which is a crucial neurotrophin associated with plasticity and neuronal survival (Brunoni et al., 2008; Lee and Kim, 2010); the assessment of neurotransmitter concentrations in the brainstem, an important brain locus for monoaminergic transmission and which is implicated in mood disorders (Sasaki et al., 2008), as well as highly influenced by the gut microbiota (Strandwitz, 2018); and the response to acute stress through the determination of plasma corticosterone, the main rodent stress hormone (de Kloet et al., 2005; Joels et al., 2018). Finally, we evaluated the consequences of MS and diet intervention with polyphenols on the gut microbiota abundance and through the determination of short-chain fatty acids (SCFAs), microbial metabolites thought to play a critical role in gut-brain communication (Dalile et al., 2019; van de Wouw et al., 2018). Together, findings from this study have the potential to provide new insights into the potential therapeutic effects of polyphenols and the role of the microbiota-gut-brain axis in stress-related disorders, and add an important direction to future dietary advice on optimal nutrition for mental health and to counter the enduring impact of early life adversity.

2. Methods

2.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork. Pregnant Sprague Dawley dams weighing 250–300 g were bred in-house in the Biological Services Unit facility, University College Cork. The pups were housed with their mothers in plastic cages (15 × 22 × 9 cm) in a temperature and humidity controlled room on a 12-h light, 12-h dark cycle (lights on from 7.00–19.00 h). Food and water were available ad libitum.

2.2 Drugs

Quercetin (Q4951) was purchased from Sigma. Xanthohumol (A-4-2014) was provided by Hopsteiner, GmbH (Mainburg, Germany). Phlorotannin-rich extract from *Fucus vesiculosus* (Gite et al., 2019) was obtained from National University of Ireland, Galway (Galway, Ireland). All diets were prepared by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany). The resulting grain based chows were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids).

2.3 Maternal separation procedure

MS was performed as previously described (O'Mahony et al., 2009; Pusceddu et al., 2015). Briefly, pups were separated from their mother as a whole litter and placed into plastic cages maintained at 30 – 33 °C in a separate room to prevent communication through ultrasonic vocalisation (Hofer et al., 1994). Following the 3-hour separation, pups were returned to their original home cage with their mother. This procedure was repeated each day (9.00am–12.00pm) from post-natal day (PND) 2 through PND12. NS-Control rats consisted of non-handled pups, left untouched by the experimenter, and with their respective mothers. After

postnatal day 12, pups were left undisturbed except for routine cage cleaning every two days. At weaning, male rats were group-housed (2 – 4) in large cages.

2.4 Treatments

The rats were randomly assigned into five different experimental groups [1] NS-Control diet (n = 12); [2] MS-Control diet (n = 12); [3] MS-Phlorotannins 0.03% (n = 10); [4] MS-Xanthohumol 0.015% (n = 10); [5] MS-Quercetin 0.03% (n = 10). Dietary intervention of polyphenols, delivered ad libitum in food, began once the animals were eight weeks old and continued for eight weeks. The concentrations for the polyphenols tested were calculated based on doses previously reported in animal models and considered the average daily food intake and body weight of Sprague Dawley rats aged between 9 and 16 weeks (Laaksonen et al., 2013). Estimated doses are as follows; quercetin 20 mg/kg/day (Haleagrahara et al., 2009); xanthohumol 10 mg/kg/day (Ceremuga et al., 2013); phlorotannins 20 mg/kg/day (Ahn et al., 2017). In the interest of reduction in the 3Rs a number of other interventions were also run contemporaneously with the control and treatment groups used here (Egerton *et al*. unpublished).

2.5 Elevated plus maze

The elevated plus maze (EPM) is one of the most commonly used rodent tests for assessing anxiety and was performed as previously described (Cryan et al., 2004; Pusceddu et al., 2015). Briefly, the maze consisted of two open arms (51 × 10 cm; 5 lux) and two enclosed arms (51 × 10 × 41 cm) that all extended from a common central platform (10 × 10 cm). The apparatus was elevated 55 cm above the floor on a central pedestal. Animals were habituated to the testing room for 30 min prior experiment. At week 12, animals were placed in the centre of the maze facing an open arm to begin. Animal behaviour was recorded for 5 min. Frequency of open and closed arms entries were scored, as well as percentage time spent in each arm.

2.6 Open field test

The open field test (OFT) is commonly used as a mechanism to assess anxiolytic effects of compounds (Seibenhener and Wooten, 2015). Briefly, at week 13 rats were placed in the centre of a white open field arena (60 × 40 cm; 60 lux) and observed for 10 min. Animals were habituated to the test room for 30 min prior to the experiment. All trials were conducted between 9.00am and 1.00pm. The arena was cleaned with 70% ethanol to avoid smell cues between each trial. At the end of each trial, animals were returned to their cages. Distance moved, velocity, percentage of time spent in inner zone, and frequency of inner zone entries were analysed and recorded using a tracking system (Ethovision XT 13, Noldus).

2.7 Forced swim test

The forced swim test (FST) is the most widely used model for predicting antidepressant activity in rodents, and increased immobility in this test is generally considered to reflect a state of behavioural despair (Porsolt et al., 1978). Briefly, at week 15 a modified rat FST protocol (Slattery and Cryan, 2012) was used to determine the antidepressant effects of polyphenols in rats. On day one, rats were placed individually in glass cylinders (H: 45 cm; D: 20 cm) filled with water to a depth of 30 cm at 24±1 °C for a 15 min pre-test period. The rats were removed from the water, dried and placed in their home cage. The cylinders of water were changed between each trial. 24 hours after the pre-test, the rats were again placed in the swim apparatus for 5 min and behaviours were monitored from above with a video camera for subsequent analysis. Behaviours rated include immobility, climbing and swimming (scoring of behaviours was blind to the experimental conditions). The 5-min session was scored using a time-sampling technique, whereby the predominant behaviour in each 5-s period of the 300-s trial was recorded. Climbing behaviour consisted of upward-directed movements of the forepaws along the side of the cylinder. Swimming behaviour was defined as movement (usually horizontal)

throughout the cylinder. The rat was considered to be immobile when the only activity observed was that which was required by the rat to keep its nose above water.

2.8 Plasma corticosterone determination

Blood sample collection was performed as previously described (Pusceddu et al., 2015). Briefly, blood samples were collected on day one of FST via a tail-tip incision at five different time points: immediately before (baseline), 30 min, 60 min, 90 min and 120 min after the test was started. Approximately 200 µl of blood was collected in a tube containing 10 µL of EDTA 0.1 M to avoid coagulation. Blood plasma was obtained by centrifugation at 3500 × *g* at 4 °C for 15 min. Corticosterone levels were measured using the Corticosterone EIA kit (Enzo) according to the manufacturer instructions, and absorbance signal was detected with a conventional plate reader (Synergy HT, Biotek).

2.9 Plasma BDNF measurement

Immediately after sacrifice, trunk blood was collected in EDTA Vacutainer tubes. Blood plasma was obtained by centrifugation at 3500 × *g* at 4 °C for 15 min. Protein levels of brain-derived neurotrophic factor (BDNF) were determined using an electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer's protocol. BDNF levels were determined and analysed using the MSD QuickPlex SQ 120 Instrument.

2.10 Brain monoamines concentration

The monoamine neurotransmitters noradrenaline (NA), serotonin (5-HT), dopamine (DA) and their metabolites 5-HIAA and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in the brainstem using high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Clarke et al., 2012; Pusceddu et al., 2015). Briefly, samples were homogenised in mobile phase (consisting in 0.1 M citric acid, 0.1 M sodium dihydrogen

phosphate monohydrate, 5.6 mM 1-octanesulfonic acid, 0.01 mM EDTA, 11.1% (v/v) methanol, and 0.1 µg/mL of N-Methyl 5-HT as internal standard and adjusted to pH 2.8). Then samples were centrifuged 14000 *g* for 15 min at 4 °C, and 20 µL of this supernatant was injected onto the HPLC system (consisting in a CBM-20A system controller, a EC3000 Recipe amperometric detector, a LC-20AD XR pump, a CTO-20A column oven at 30 ^{0}C, a SIL-20AC XR autosampler, and a Prominence DGU-20A3 degasser). A reverse-phase column (Kinetex 2.6u C18 100A 100 mm X 4.6 mm, Phenomenex) was employed in the separation using a flow rate of 0.9 mL/min. Each neurotransmitter was identified through their characteristic retention times and their concentration was determined using the ratios of peak heights of analyte versus internal standard provided by the LabSolutions software (Shimadzu). Results were expressed as nanograms of neurotransmitter per grams of fresh tissue.

2.11 Gut microbiota 16S rRNA sequencing

Microbial DNA was isolated from frozen faecal samples using the QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. DNA concentration and quality was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S metagenomic sequencing library protocol, and PCR reactions were performed with the KAPA HiFi HotStart PCR Kit (KAPA Biosystems). PCR products were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter Life Sciences). This was followed by indexing PCR which attached Nextera XT barcodes and Illumina sequencing adapters to the 5′ overhangs and another round of AMPure XP clean-up. Finally, samples were sequenced on the MiSeq™ System (Illumina®), using a 2 x 250bp cycle kit, following standard Illumina sequencing protocols.

2.12 Short chain fatty acid determination

The SCFAs acetate, propionate, butyrate, and valerate, as well as the total branched chain fatty acids (BCFAs) were measured in caecal content using gas chromatography flame ionisation detection (GC-FID) as previously reported (van de Wouw et al., 2018). Briefly, samples were vortexed with Milli-Q water (1:10 w/v), left to stand for 10 min at room temperature, and then centrifuged at 14000 *g* for 5 min. The supernatant was filtered (0.2 µm) before transfer to a GC glass vial, and 2-ethylbutyric acid (Sigma) was added as internal standard. SCFA concentrations were measured using a Varian CP-3800 GC flame-ionization system, fitted with a Zebron ZB-FFAP column (30 m × 0.32 mm × 0.25 µm; Phenomenex) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was used as the carrier gas at a flow rate of 1.3 ml/min. The initial oven temperature was set at 100 °C for 0.5 min, raised to 180 °C at 8 °C/min and held for 1 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min. The temperature of the injector and the detector were set at 240 °C and 250 °C respectively. A standard curve made from a standard mix of acetic acid, propionic acid, n-butyric acid and iso-butyric acid (Sigma) at seven concentrations. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain calibration.

2.13 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean ± SEM. MS-control group and NS-control group were compared using independent T-test to assess the MS effect. All MS groups were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A *p*-value of 0.05 was considered statistically significant. FLASH was used to assemble paired-end reads. Further processing of paired-end reads including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (version 1.9.0).

Denoising, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST, and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Statistical microbiome analysis was carried out in R (version 3.6.1) with Rstudio (version 1.2.1335). OTUs unknown on a genus level were excluded, as well as OTUs available in two or fewer samples. The ALDEx2 library (Fernandes et al., 2014) was used to compute the centred log-ratio transformed values of the remaining taxa. For principal component analysis, a pairwise implementation of the adonis() PERMANOVA function in the vegan library (Oksanen et al., 2017) followed by the Bonferroni-Holm correction was used to test for difference in β-diversity in terms of Aitchison distance. Differential abundance was assessed using a pairwise implementation of the aldex.test() function, followed by Benjamini-Hochberg correction. In these cases, a q-value < 0.1 was considered significant. α-diversity was computed using the iNEXT library (Hsieh et al., 2016).

2.14 Functional prediction of Gut-Brain modules

The Piphillin webservice (Iwai et al., 2016) was used to infer the functional metagenome per sample in terms of KEGG orthologues. Next, these KEGG orthologues were processed using the omixer library in R (Darzi et al., 2016) in order to calculate abundance of gut-brain-modules (GBMs) (Valles-Colomer et al., 2019) and gut-metabolic modules (GMMs) in these samples. Then, the same implementation from ALDEx2 was used to assess differential abundance. Scripts are publicly available on GitHub: https://github.com/thomazbastiaanssen/Tjazi doi: 10.5281/zenodo.1480804

3. Results

3.1 Polyphenols reversed MS-induced depressive-like behaviours

To investigate the therapeutic effects of the dietary interventions with polyphenols from MS-induced behavioural despair, animals were subjected to a battery of behavioural tests to examine depressive- and anxiety-like behaviours. Firstly, animals did not differ in terms of body weight across the different experimental groups throughout the duration of the treatment (Fig. 1B and C). In FST, analysis yielded a significant effect of MS compared to NS-control group on the time spent immobile (t_{22} = -2.349; p = .028) and swimming (t_{22} = 2.611; p = .016) (Fig. 2A). MS animals exhibited improved depressive-like behaviours with xanthohumol; moreover, quercetin and phlorotannins significantly decreased immobility time in the FST ($F_{3,36}$ = 4.425; p = .05 and p = .002 respectively). In addition, treatment with phlorotannins increased swimming time compared to the MS-control group ($F_{3,36}$ = 2.984; p = .008) (Fig. 2A).

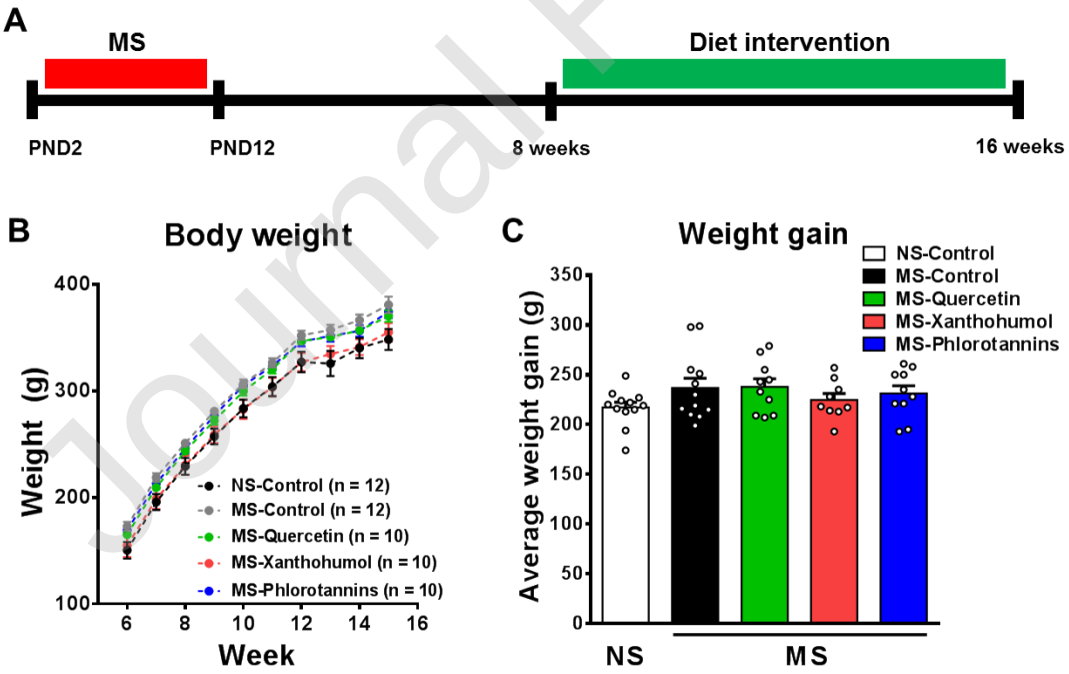

Figure 1 Diet intervention with polyphenols did not affect body weight. (A) *Schematic representing the experimental timeline.* ***(B)*** *Body weight was measured weekly from 6-week old until the end of the diet intervention.* ***(C)*** *The weight gain was calculated as the difference between the first body weight record (6 weeks) and the last measurement (15 weeks).*

3.2 Polyphenols showed anxiolytic potential in MS animals

MS-induced anxiety-like behaviour in the OFT by significantly reducing the time spent in the centre (t_{21} = 2.156; p = .025), as well as in the number of entries in the centre of the arena (t_{21} = 2.855; p = .009) (Fig.2D and E). Administration of quercetin in MS rats resulted in a significant increase in the number of entries in the open arms compared to the MS-control group ($F_{3,38}$ = 2.714; p = .040), which was associated with an anxiolytic effect (Fig. 2G). Interestingly, treatment with phlorotannins ameliorated MS-induced anxiogenic effects in both, time in centre ($F_{3,37}$ = 2.297; p = .025) and in entries into the centre ($F_{3,37}$ = 2.405; p = .025). However, no differences were found between NS-control and MS animals during the EPM (Fig. 2F and G).

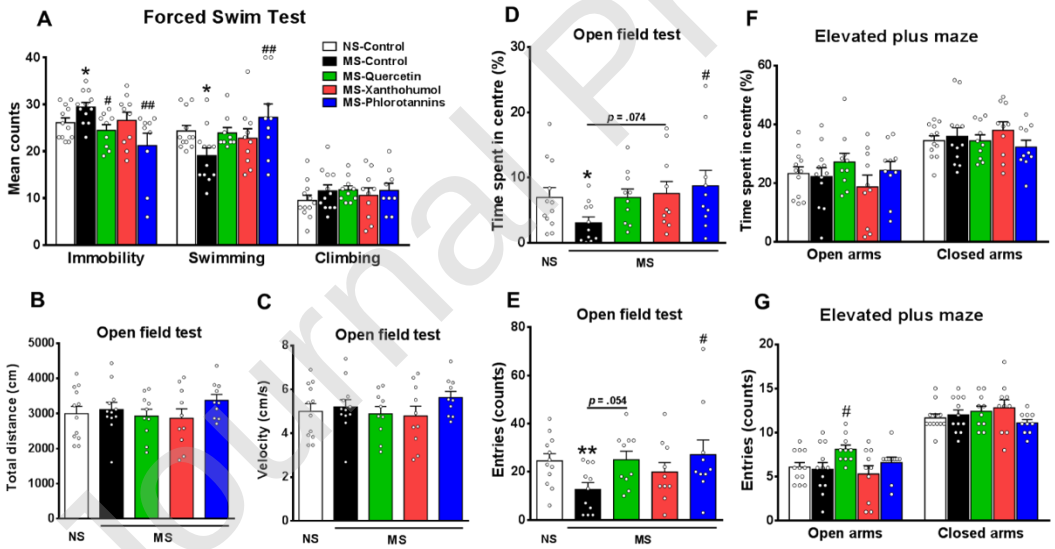

Figure 2 Treatment with polyphenols induced antidepressant- and anxiolytic-like effects in MS rats. (A) *MS-induced increased immobility in the FST is prevented through treatment with quercetin and phlorotannins, while reduced swimming time caused by MS is reversed only by phlorotannins treatment.* ***(B – C)*** *Polyphenolic diets nor MS produced changes in locomotor activity.* ***(D – E)*** *Phlorotannins treatment produced a significant increase in the time spent in centre and number of centre entries when is compared to MS-control group in the OFT.* ***(F – G)*** *However, MS animals did not show anxiety-like behaviours in the EPM. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01 versus 'vehicle' groups; #p < 0.05; ##p < 0.01 versus 'CORT' groups).*

3.3 Xanthohumol prevented the exacerbated corticosterone production in MS rats after acute stress

To determine the role of the HPA axis in MS-induced depressive- and anxiety-like behaviours, the concentration of corticosterone in plasma was measured at different time points after an acute stress (Fig. 3A). Indeed, the corticosterone production was close to being statistically increased in MS-control relative to the NS-control group as revealed by the area under the curve (AUC) of corticosterone response (t_{20} = -1.949; p = .065) (Fig. 3B). Interestingly, dietary intervention with xanthohumol in MS animals induced a significant reduction in corticosterone AUC compared to the MS-control group ($F_{3,34}$ = 3.827; p = .010) (Fig. 3B). In addition, all polyphenolic treatments induced lower baseline levels of plasma corticosterone compared to MS-control group ($F_{3,36}$ = 3.979; quercetin p = .080; xanthohumol p = .006; phlorotannins p = .011).

3.4 MS-induced plasma BDNF reduction was reversed by xanthohumol treatment

BDNF is a critical modulator of neuroplasticity and survival, abundant in the brain and periphery, including serum and plasma (Lee and Kim, 2010). Preclinical and clinical studies have demonstrated that chronic stress and depressive status reduces BDNF expression (Gonul et al., 2005; Russo-Neustadt et al., 2001). Indeed, MS rats showed lower levels of plasma BDNF compared to NS animals (t_{19} = 2.672; p = .015), and this effect was significantly prevented by xanthohumol treatment ($F_{3,36}$ = 1.748; p = .047) (Fig. 3D).

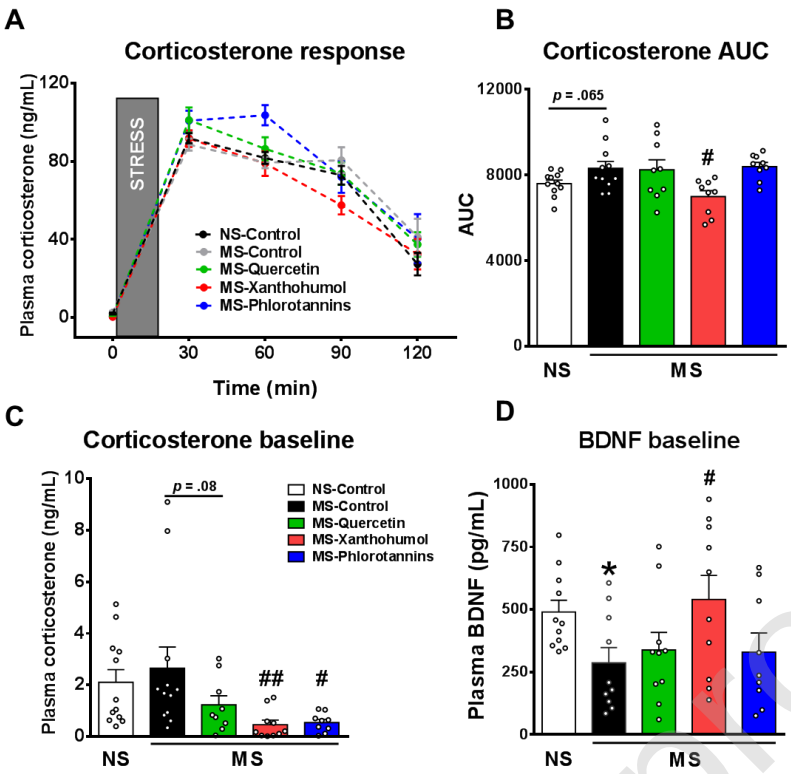

Figure 3 Xanthohumol treatment prevented corticosterone elevation and BDNF reduction in MS rats. (A) *Corticosterone levels in plasma rise after rats are exposed to an acute stress.* ***(B)*** *MS-induced increase in corticosterone release is abolished by treatment with xanthohumol.* ***(C)*** *Baseline levels of corticosterone of rats treated with xanthohumol and phlorotannins are significantly lower compared to MS animals.* ***(D)*** *Rats treated with xanthohumol displayed higher levels of plasma BDNF compared to the MS-control group. Plasma corticosterone was determined using ELISA, and BDNF determination was performed with MSD system. Results are expressed as the mean ± SEM (*p < 0.05 versus 'vehicle' groups; #p < 0.05; ##p < 0.01 versus 'CORT' groups).*

3.5 MS induced decreased levels of DA and 5-HIAA in brainstem

To further determine the effects of early life stress on neurochemistry, and its potential implication on the antidepressant and anxiolytic effects of polyphenols, monoamine neurotransmitter concentration was measured in the brainstem. MS produced a significant reduction of DA and 5-HIAA levels ($t_{20} = 6.121$; $p = .000$ and $t_{22} = 3.934$; $p = .001$ respectively) (Fig. 4B and D), reduced 5-HT turnover ($t_{21} = 3.519$; $p = .002$) (Fig. 4C), and increased DA turnover ($t_{22} = -2.153$; $p = .047$) (Fig. 4F). In contrast, treatment with phlorotannins increased the levels of NA and 5-HT compared to the MS-control group (Fig. 4A and G).

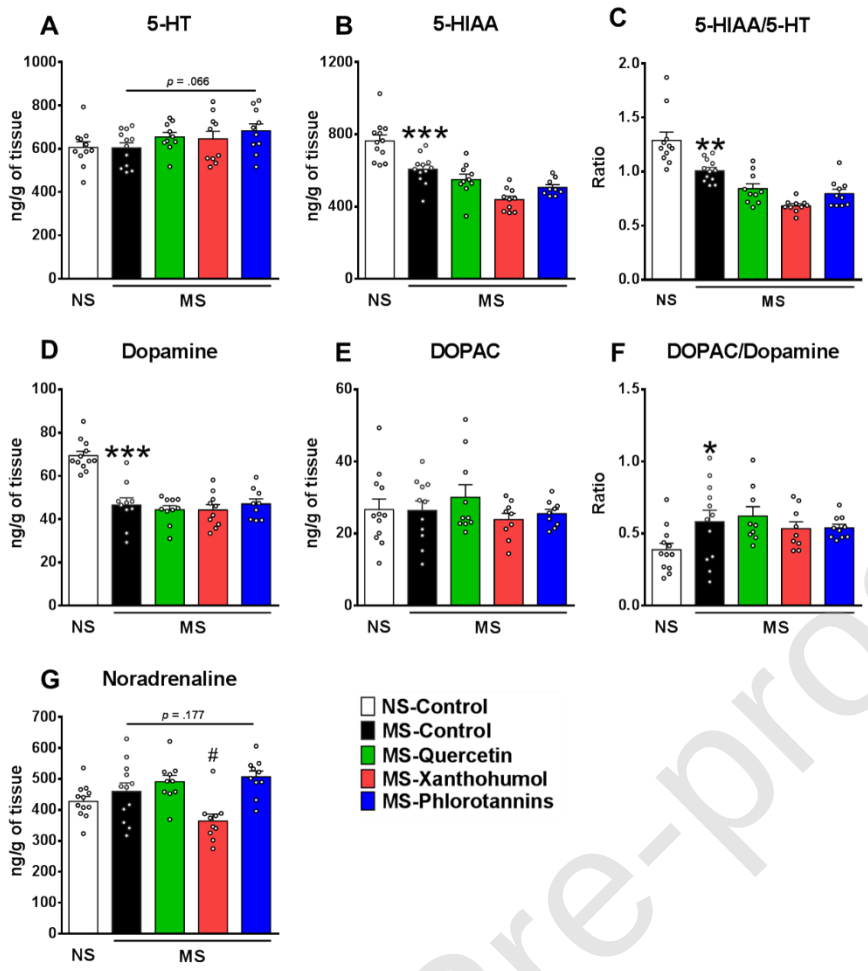

Figure 4 Polyphenols did not prevent MS-induced reductions in brainstem dopamine and 5-HIAA. *Monoamine neurotransmitters were measured in the brainstem via HPLC. MS animals show depleted concentrations of dopamine and 5-HIAA compared to NS-control rats. Phlorotannins diet intervention exerted an increase of noradrenaline and 5-HT MS animals, although not significant. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

3.6 MS and dietary treatments induced changes in gut bacterial diversity

To define whether the experimental treatments also altered gut microbiota diversity and bacterial abundance, α- and β-diversity analyses were performed. Although no differences in richness were found using the Chao1 α-diversity metric (Fig. 5A), Shannon entropy and Simpson index both indicate that MS rats treated with phlorotannins showed reduced diversity within this group compared to the MS-control experimental group (p = .072 and p < .05, respectively) (Fig. 5B and C). In other words, while the total estimated amount of OTUs did not differ, the microbial ecosystem of animals treated with phlorotannins were distributed less

evenly. On the other hand, principal component analysis (PCA) to measure the diversity among groups, indicated that MS and NS control groups were significantly different from each other ($F_{4,47}$ = 2.012; p = .046) (Fig. 5D). In addition, treatments with quercetin, xanthohumol and phlorotannins also produced significant changes in β-diversity in terms of Aitchison distance compared to MS-control group ($F_{4,47}$ = 2.012; p = .004; p = .045; p = .046, respectively) (Fig. 5D).

3.7 Changes in the gut microbiota composition correlated with MS status and polyphenolic diets

Alteration of the gut microbiota composition has been associated with different mental disorders, including major depression and other stress-related psychiatric disorders (Cryan and Dinan, 2012). Thus, we examined the differences in the gut microbiota composition of maternally separated rats. Significant differences in terms of the relative abundance between MS-control and NS-control animals were found in 5 specific bacteria based on effect size (*Streptococcus; Ruminococcus; Parabacteroides; Rothia; Christensenellaceae*; q < .1) (Fig. 5E). On the other hand, dietary interventions with quercetin and xanthohumol in MS rats induced significant changes in the abundance of other bacteria genera when compared to the MS-control group. Specifically, quercetin produced a significant increase of *Enterorhabdus* (q < .1), while xanthohumol exerted changes in the abundance of *Asteroplasma*, *Lachnospiraceae*, and *Coprococcus* (q < .1) (Fig. 5E).

3.8 Treatment with phlorotannins and xanthohumol restore MS-induced changes in bacteria associated with microbiota-gut-brain pathways

To investigate the implications of MS-induced changes in gut microbiota composition on metabolic pathways associated with the microbiota-gut-brain axis, we performed a functional prediction based in previously described GBMs (Valles-Colomer et al., 2019). MS significantly changed the abundance of bacteria linked to 8 GBMs in terms of effect size, including

tryptophan degradation, quinolinic acid metabolism, nitric oxide metabolism, and p-cresol synthesis compared to NS-control group ($q < .1$) (Fig. 5F). Intriguingly, although quercetin did not alter any relevant bacteria, xanthohumol and phlorotannins treatment restored most of the changes produced by MS in these bacteria.

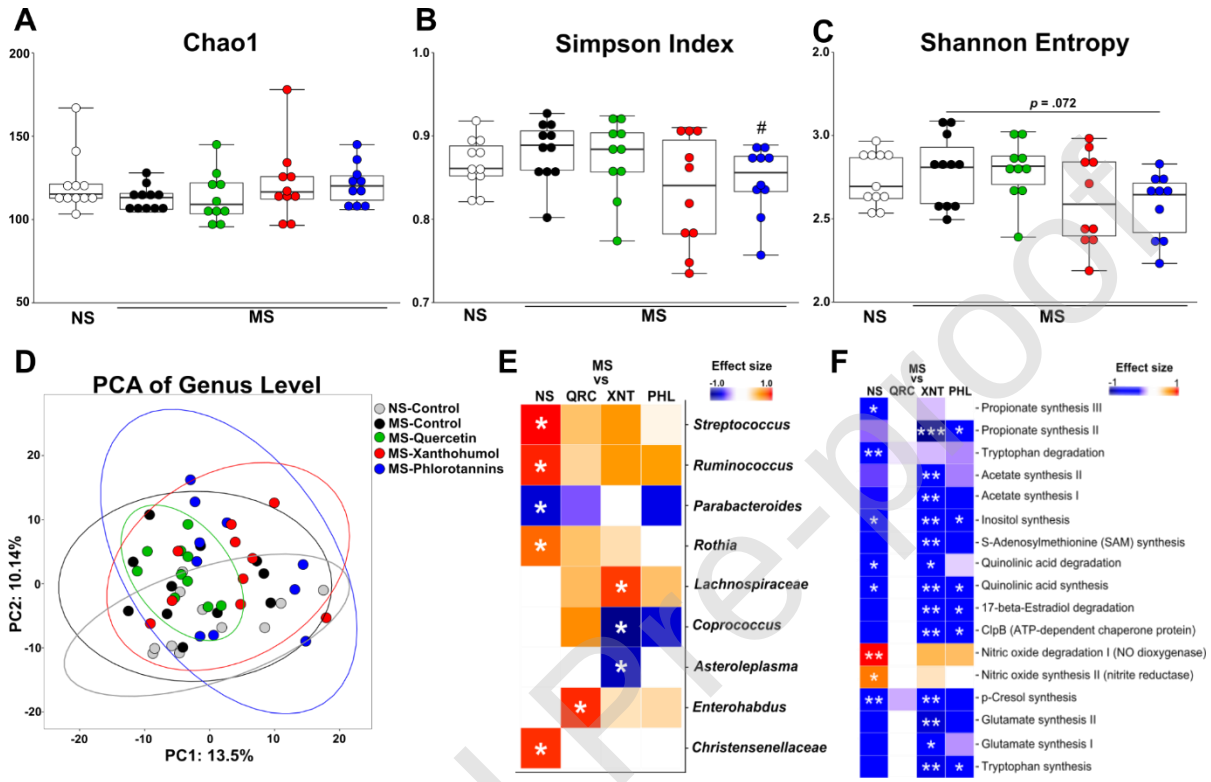

Figure 5 MS and polyphenolic diets induced significant changes in gut microbiota composition and diversity. ***(A – C)*** *Chao1, Simpson index and Shannon entropy were used as estimators of bacterial α-diversity.* ***(D)*** *Principal component analysis of genus level was performed to estimate the β-diversity between experimental groups.* ***(E)*** *Bacterial abundances were significantly altered in MS rats in terms of effect size ($q < .1$). In contrast, xanthohumol and quercetin changed other bacteria compared to MS animals.* ***(F)*** *Functional prediction of GBMs was utilised to detect potential microbiota-gut-brain pathways affected by MS or dietary treatments. Colours represent effect size, only microbiome features found to be significantly different in at least one comparison are shown (*$q < .1$; **$q < .05$; ***$q < .01$ vs MS-control group).*

3.9 Xanthohumol prevented MS-induced reduction of intestinal SCFAs

To determine whether the observed changes in the gastrointestinal microbiota composition and diversity correlate with alteration in SCFA production, the levels of acetate, propionate, butyrate, valerate were determined in caecal content. Interestingly, maternal separation induced a significant reduction of acetate (t_{22} = 2.409; p = .025), propionate (t_{22} = 2.988; p = .01), isobutyrate (t_{21} = 3.354; p = .006), isovalerate (t_{21} = 2.779; p = .016), total SCFAs (t_{21} = 2.228; p = .037), and total BCFAs (t_{21} = 3.181; p = .008). In contrast, phlorotannin treatment significantly reversed the MS-induced propionate reduction ($F_{3,38}$ = 4.646; p = .022), and exerted positive effects on isobutyrate, valerate and total levels of BCFAs.

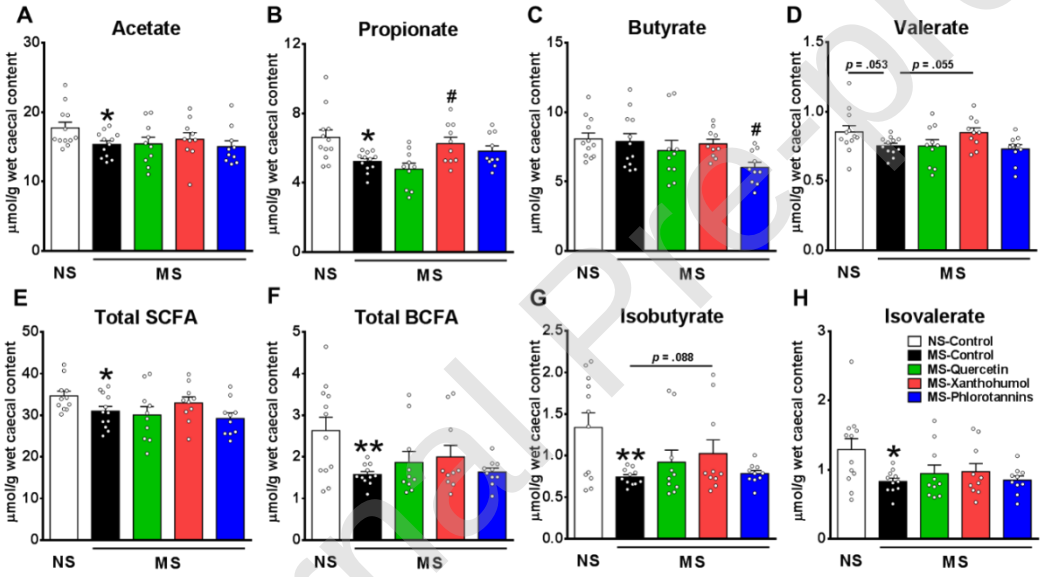

Figure 6 MS rats exhibited lower levels of gut microbiota-derived metabolites (A – H). *MS induced significant reduction of gut microbiota-derived metabolites including acetate, propionate, isobutyrate and isovalerate, as well as decreased total short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA). Xanthohumol treatment ameliorated MS-induced propionate decrease and tends to improve isobutyrate and valerate levels. Fatty acid determination was performed through HPLC in caecal content. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

4. Discussion

There has been increasing attention given to the potential of nutritional approaches to ameliorate the effects of stress (Lakhan and Vieira, 2008; Marzola et al., 2013; Rechenberg and Humphries, 2013). In the present study, we tested different naturally-derived polyphenols as potential therapeutic strategies for depression and anxiety associated with early life trauma. Indeed, the polyphenols quercetin, xanthohumol and phlorotannins exert varying degrees of antidepressant- and anxiolytic-like responses in rats subjected to MS. Moreover, dietary interventions also modified gut microbial composition and diversity, suggesting that their therapeutic effects could be associated with the microbiota-gut-brain axis.

The MS rat is an excellent model to study the negative effects of early life stress on brain function and structure, which are associated with the development of depression and anxiety (O'Mahony et al., 2011; Vetulani, 2013). MS in rats induce a robust depressive-like phenotype in adult animals, including changes in gut microbiota, dysregulation of the HPA axis, and an imbalance in neurotransmitter levels (Aisa et al., 2007; Daniels et al., 2004; Desbonnet et al., 2010; Liao et al., 2019; O'Mahony et al., 2009). Furthermore, we demonstrated that all polyphenolic treatments tend to reverse these depressive-like behaviours. In particular, the phlorotannin-enriched diet produced a significant improvement in immobility and swimming behaviour in the FST compared to the MS-control group. Although the effect of polyphenols has been recently investigated in animal models of stress (Kwatra et al., 2016; Samad et al., 2018; Yang et al., 2017), to our knowledge there is no data on the effects of dietary intervention with polyphenols in animal models of early life stress *per se*.

Regarding anxiety, quercetin administration exerted a significant anxiolytic effect in MS animals, resulting in an increase in the number of entries into the open arms of the EPM.

Similarly, quercetin- and xanthohumol-enriched diets tend to induce anxiolytic effects in the OFT, while phlorotannin treatment revealed a significant improvement. Although the concept of a potential therapeutic effect of polyphenols in animal models of stress is not completely new (Anjaneyulu et al., 2003; Aubry et al., 2019; Hurley et al., 2014), the neurobehavioural effects of polyphenols in a model of early-life stress have not to our knowledge been examined previously.

We further investigated the role of the HPA axis in the therapeutic effects of polyphenol administration. Accumulated lines of evidence indicate that depressive or chronically stressed patients have an over activated HPA axis (Keller et al., 2017; Pariante and Lightman, 2008). Similarly, animals subjected to chronic stress possess a dysregulated HPA axis and increased baseline levels of glucocorticoids (O'Mahony et al., 2011; Uschold-Schmidt et al., 2012). Indeed, we demonstrated that the dietary intervention with xanthohumol significantly reduced the exacerbated production of corticosterone in MS animals.

Next, we demonstrated that treatment with xanthohumol prevented the MS-induced reduction in plasma BDNF. BDNF has strongly been implicated in antidepressant activity, and plasma BDNF has been shown to reflect aspects of that centrally and to be a biomarker of antidepressant effect (Lee and Kim, 2010; Sen et al., 2008; Woelfer et al., 2019). In addition, a positive correlation of BDNF levels between blood and brain has been demonstrated in rats (Harris et al., 2016; Karege et al., 2002; Sartorius et al., 2009), and a substantial amount of the circulating BDNF has been proposed to originate from the CNS itself (Dawood et al., 2007; Krabbe et al., 2007; Rasmussen et al., 2009). Although the possible pathways involved in BDNF rescue must be further investigated, it is tempting to speculate that the positive effects of xanthohumol on behaviour could be partly mediated by normalising BDNF expression.

The relationship between stress and the gut microbiota is gaining a lot of attention (Bastiaanssen et al., 2020; Foster et al., 2017). Additionally, we have demonstrated that MS is able to induce strong changes to the gut microbiota in terms of composition and diversity which is in line with previous reports (Moussaoui et al., 2017; O'Mahony et al., 2009). Although, we did not detect changes at the α-diversity level, analysis of β-diversity revealed that MS groups treated with polyphenols differ from the MS control group. We followed this up by assessing differential abundance of bacterial genera between the treatment groups. Notably, only xanthohumol and quercetin treatments produced significant changes in certain bacterial genera in MS rats, suggesting that some polyphenol-enriched diets have the potential to modify bacterial composition in the gastrointestinal system. Several studies have demonstrated the capacity of polyphenolic intake to shape the gut microbiota (Etxeberria et al., 2013; Ozdal et al., 2016). The fact that all types of polyphenol intake were found to alter β-diversity compared to MS control, but only xanthohumol and quercetin yielded differences in the abundances of specific genera may suggest that polyphenols induce a general shift in the microbial composition, which may be indicative of a change in functionality in the microbiome.

Therefore, we performed a functional prediction of the gut metagenome and used this to infer the abundance of GBMs, metabolic modules that are involved in the microbiota-gut-brain axis (Valles-Colomer et al., 2019). Indeed, the analysis predicted that MS is able to increase the abundance of GBMs associated with the modulation of several pathways altered in depression and other neuropsychiatric disorders, including metabolism of tryptophan (Curzon and Bridges, 1970; Oxenkrug, 2010), inositol (Coupland et al., 2005), p-cresol (Persico and Napolioni, 2013), quinolinic acid (Steiner et al., 2011), nitric oxide (Dhir and Kulkarni, 2011), and glutamate (Murrough et al., 2017; Sanacora et al., 2012). Interestingly, treatment with xanthohumol and phlorotannins reversed these predicted MS-induced changes, suggesting that restoration of these GBMs may partially explain their positive effects in behaviour. An

important limitation due to the nature of 16S sequencing is that functional analysis can only be inferential. Future metabolomics-based studies should address this experimentally.

In addition, our data revealed that MS rats exhibited decreased production of SCFAs compared with the NS-control group. We detected a significant reduction of acetate, propionate, isobutyrate, and isovalerate. The production of SCFAs is highly associated with certain bacterial populations in the gut, and there is common agreement surrounding the impact of SCFAs on human metabolism and health (Morrison and Preston, 2016). Indeed, it is widely accepted that SCFAs play a critical role in gut-microbiota-brain communication, and consequences for mental health and behaviour (Dalile et al., 2019; Stilling et al., 2016). A preclinical study showed that a depression-associated microbiota makeup can impact SCFA production (Kelly et al., 2016), and that SCFAs can reverse the enduring effects of stress in a mouse model (van de Wouw et al., 2018). In our study, we demonstrated that treatment with xanthohumol specifically prevented the reduction of propionate in MS rats. Since the xanthohumol diet intervention induced acute changes in bacterial composition of the MS gut microbiota, we presume that the changes observed in propionate levels could be a product of improved microbial metabolism.

In conclusion, our present work confirmed that the naturally derived polyphenols xanthohumol, quercetin and phlorotannins can alleviate depressive- and anxiety-like behaviours in the rat MS model. We further found that treatment with xanthohumol prevented exacerbated production of corticosterone after acute stress in MS animals, and reversed MS-induced plasma BDNF depletion. In addition, our data revealed that MS-induced behavioural despair correlated with significant changes in bacterial composition and diversity, alteration of predicted microbiota-gut-brain pathways, and reduced SCFA production. Although all polyphenols caused changes in diversity, only xanthohumol induced significant changes in several bacterial taxa and

prevented the reduction of propionate in MS rats. Taken together, our findings present evidence of the therapeutic properties of polyphenols and provide a novel insight into the potential mechanisms underlying their antidepressant effect.

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